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Attenuation of HLA-DR Expression by Mononuclear Phagocytes Infected with Mycobacterium tuberculosis Is Related to Intracellular Sequestration of Immature Class II Heterodimers

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MHC class II expression was examined in macrophages infected with Mycobacterium tuberculosis. IFN-γ increased the surface expression of class II molecules in THP-1 cells and this was markedly reduced in cells infected with M. tuberculosis. Despite this effect, steady state levels of HLA-DRα, HLA-DRβ, and invariant (Ii) chains were equivalent in control and infected cells. Metabolic labeling combined with pulse-chase experiments and biochemical analysis showed that the majority of class II molecules in infected cells became resistant to endoglycosidase H, consistent with normal Golgi processing. However, results of intracellular staining and dual color confocal microscopy revealed a significant defect in transport of newly synthesized class II molecules through the endocytic compartment. Thus, compared with findings in control cells, class II molecules in infected cells colocalized to a minimal extent with a lysosomal-associated membrane protein-1+ endosomal compartment. In addition, in contrast to control cells, class II molecules in infected cells failed to colocalize with endocytosed BSA under conditions where this marker is known to label late endosomes, lysosomes, and the MHC class II compartment. Consistent with defective transport along the endocytic pathway, the maturation of SDS-stable class II αβ dimers—dependent upon removal of Ii chain and peptide loading of class II dimers in the MHC class II compartment—was markedly impaired in M. tuberculosis-infected cells. These findings indicate that defective transport and processing of class II molecules through the endosomal/lysosomal system is responsible for diminished cell surface expression of MHC class II molecules in cells infected with M. tuberculosis. The Journal of Immunology, 1998, 161: 4882–4893.

Tuberculosis is a life-threatening bacterial disease with an estimated eight million new cases and three million deaths reported worldwide each year. The incidence of tuberculosis is particularly high in developing countries, and among infectious diseases, it is the leading cause of death worldwide (1–3). Once thought to be nearing eradication in developed countries, tuberculosis has undergone a dramatic resurgence in parallel with the HIV pandemic (4–6). Thus, new strategies for the prevention and treatment of tuberculosis are urgently needed, and parallel with the HIV pandemic (4–6).

4882–4893.

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2 Z.H. was a visiting scholar from the University of Fes, Fes, Morocco.

3 Address correspondence and reprint requests to: Dr. Neil E. Reiner, the Division of Infectious Diseases, Department of Medicine, University of British Columbia, Room 452D, 2733 Heather St., Vancouver, BC, Canada, V5Z 3Z5. E-mail address: ethan@unixg.ubc.ca

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4 Abbreviations used in this paper: CIITA, class II transactivator; Ii, invariant; MHC, MHC class II compartment; Jak, Janus kinase; TGN, trans-Golgi network; CLIP, class II-associated Ii peptide; MFI, mean fluorescence intensity; ECL, enhanced chemiluminescence; DIG, digoxigenin; Endo H, endoglycosidase H; LAM, liposaccharidomannan; Ii chain, invariant chain.
Newly synthesized class II molecules and Ii chain exit the endoplasmic reticulum, traverse the Golgi and trans-Golgi network (TGN), subsequently localizing to an acidic endosomal compartment. The events that transpire in this compartment are complex and essential to ensure appropriate expression of class II dimers. Thus, in this compartment, Ii undergoes sequential proteolysis (22, 23), and the final product of Ii degradation, a peptide referred to as CLIP (class II-associated Ii peptide), is removed and εβ dimers are loaded with antigenic peptide (24). HLA-DM, a nonclassical MHC class II molecule, stably associates with CLIP-associated εβ dimers and catalyzes the exchange of CLIP with cognate peptide (25, 26). Mature, peptide-loaded, SDS-stable class II dimers are then transported to the cell surface. Removal of CLIP and peptide loading is believed to occur in an endosomal/lysosomal compartment referred to as the MHC class II compartment (MIIC).

This study examined the basis for diminished IFN-γ-induced cell surface expression of class II molecules in M. tuberculosis-infected human mononuclear phagocytes. The results show that infection has no apparent effect on signaling for expression of CIITA and DRA genes. Rather, diminished class II expression is related to defective trafficking of apparently immature class II molecules from the TGN through the endocytic compartment.

Materials and Methods

Reagents and chemicals

RPMI 1640 and HBSS were obtained from the Terry Fox Laboratory, Vancouver, British Columbia, Canada. PMA, PMSF, pepstatin A, aprotinin, leupeptin, and dextran sulfate were from Sigma Chemical Co. (St. Louis, MO). Latex particles (1.05 μm diameter) were from Polysciences, Inc. (Warrington, PA). Anti-phosphotyrosine mAb 4G10 and rabbit antiserum to Jak1 and Jak2 were from Upstate Biotechnology (Lake Placid, NY). Rabbit antiserum to STAT1 was kindly provided by Dr. Andrew Lanier, Food and Drug Administration (Bethesda, MD). Human rIFN-γ was a generous gift of Genentech (South San Francisco, CA).

Mycobacterium tuberculosis

A virulent strain of M. tuberculosis (Edram, TMC no. 107, Trudeau Mycobacterial Culture Collection, Saranac Lake, NY) was grown to late log phase in Proskauer and Beck medium supplemented with 0.05% Tween-80. Batch cultures were aliquoted and stored at −70°C. Representative viable counts were enumerated using a viable colony-forming unit (CFU) on Middlebrook 7H10 agar (Difco Laboratories, Detroit, MI). Prior to infection, bacteria were osonized as follows: 108 viable organisms were suspended in 1 ml of RPMI 1640 containing 50% AB-serum and rocked for 30 min at 37°C. Bacteria were then pelleted and resuspended in 1 ml of RPMI 1640 and clumps were disrupted by multiple passages through a 25-gauge needle as described (27). To evaluate the phagocytosis of M. tuberculosis by THP-1 cells, bacteria (109/ml) were labeled by incubation with 0.5 mg of FITC (Sigma) per ml in 0.1 M carbonate buffer (pH 9.0) at 37°C for 2 h. Thereafter, FITC-labeled bacteria were washed twice with PBS to remove unbound FITC, and cells were suspended in fresh Proskauer and Beck medium and kept at −70°C.

Differentiation and infection of THP-1 cells

The monocytic cell line THP-1 (ATCC, Rockville, MD) was cultured in RPMI 1640 supplemented with 10% FCS (HyClone, Logan, UT), 2 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were seeded at a density of 104/cm2 and allowed to adhere and differentiate in the presence of PMA (20 ng/ml) at 37°C in a humidified atmosphere of 5% CO2 for 24 h. Depending on the quantity of cell material needed, either in the presence of PMA (20 ng/ml) at 37°C. To quench fluorescence of noningested, but membrane-associated bacteria, cells were washed and resuspended in sodium acetate buffer (0.05 M, pH 4.5) containing 0.06% trypan blue for 5 min at 4°C. After two washes with sodium acetate buffer, the number of cells loaded with bacteria was measured by flow cytometry. Appropriate controls were included to validate the quenching of surface fluorescence.

Cell staining and flow cytometry

To measure cell surface expression of HLA-DR, control and M. tuberculosis-infected cells were incubated with anti-HLA-DR mAb (clone HL38, IgG1, Caltag Laboratories, San Francisco, CA) for 30 min, then washed twice and labeled with FITC-conjugated F(ab')2 sheep anti-mouse IgG (Sigma) for 30 min. Cells were also stained with the following mAbs: W6/32 (IgG2a, anti-HLA-class I, ATCC); IB4 (IgG2a, anti-CD18, a gift from Dr. D. Speert, the University of British Columbia, BC, Canada); and 5E9C11 (IgG1, anti-transferrin receptor, ATCC). All staining and washing procedures were performed at 4°C in HBBS containing 0.1% NaN3 and 1% FCS. To control for cell viability, cells were incubated with propidium iodide (0.5 μg/ml in staining buffer) for 10 min. The cells were then washed once and fixed in 2% paraformaldehyde in staining buffer. The combination 2% paraformaldehyde + 0.1% NaN3 killed both free and cell-associated bacteria. Cell fluorescence was analyzed using a Coulter Elite flow cytometer (Hialeah, FL). Viable cells were identified by exclusion of propidium iodide. Relative fluorescence intensities of 5000 cells were recorded as single-parameter histograms (log scale, 1024 channels, 4 decades) and the mean fluorescence intensity (MFI) was calculated for each histogram. Results are expressed as MFI index, which corresponds to the ratio: MFI of cells + specific Ab/MFI of cells + irrelevant isotype-matched IgG.

Immunoprecipitation and Western blotting

Whole cell lysates were immunoprecipitated with rabbit antiserum to Jak1, Jak2, or STAT1α and analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine mAb (4G10) as previously described (14). To assess the amount of individual proteins immunoprecipitated in each sample, after detection of bound anti-phosphotyrosine Ab, membranes were stripped, probed with either anti-Iak1, anti-Iak2, or anti-STAT1α, and developed by enhanced chemiluminescence (ECL) as described (14).

RNA isolation and RT-PCR

RNA preparation, cDNA synthesis, and PCR condition were described previously (30). Sequences of oligonucleotide primers used in PCR amplifications are as follows: DRA sense, CGA GTT CTA TCT GAA TCC CGT GGG TGG GTG GGC; DRA antisense, CGA GTT CTA TCT GAA TCC CGT GGG TGG GTG GGC; CIITA sense, CAA TCT CCT GAA GAA TGT GGA; CIITA antisense, ACG TCC ATC ACC CGG AGG GAC; DRA sense, CGA GTT CTA TCT GAA TCC CGT GGG TGG GTG GGC; DRA antisense, CCA CAC GGA GTA CTT GGC CTC AGG.

Quantification of mRNA transcripts by photometric enzyme immunoassay

Polyadenylated RNA was isolated from ribosomal RNA and transfer RNA by base-pairing between the poly(A) residues of the mRNA and a biotin-labeled oligo(dT)12–18 probe (Boehringer Mannheim kit, Montreal, Quebec, Canada) according to the manufacturer’s instructions. A second kit (Northem-ELISA, Boehringer Mannheim) was used for direct detection and quantification of DRA transcripts. In brief, mRNA samples were biotinylated in RNase-free distilled water for 1 h at 65°C and precipitated by incubation in absolute ethanol for 30 min at −70°C followed by a 30-min centrifugation at 15,000 × g. A digoxigenin (DIG)-labeled DNA probe was prepared by PCR amplification of cDNA from positive cells (THP-1 cells + IFN-γ) in the presence of DIG-UTP (Boehringer Mannheim) (35% DIG-UTP and 65% dUTP) and the sense and anti-sense oligonucleotide primers for DRA and β-actin used in the RT-PCR experiments. A DIG-chloromphenicol acetyltransferase-labeled probe (Boehringer Mannheim) was used to control for nonspecific hybridization.

Biotinylated mRNA was diluted in hybridization buffer and prewarmed to 50°C before addition of denatured (5 min, 100°C) DIG-labeled DNA probes. The hybridization mixtures were incubated for 3 h at 50°C and then transferred to streptavidin-coated microtiter plates and incubated for 5 min at 50°C. The microwells were washed and peroxidase-conjugated rabbit anti-DIG-IgG was added for 30 min at 37°C. After washing, the wells were...
FIGURE 1. Phagocytosis of FITC-labeled \(M.\) tuberculosis by THP-1 cells. A, Normal THP-1 cells or THP-1 cells differentiated with PMA (10\(^6\) cells/ml) were incubated with either nonopsonized or serum-opsonized (Ops) FITC-labeled \(M.\) tuberculosis (50 \(\times\) 10\(^9\) ml). B, Non-differentiated cells were incubated to increasing concentration of normal bacteria and PMA-differentiated cells were exposed to the same range of opsonized bacteria. After 2-h incubation at 37°C, cells were washed and surface fluorescence (noninternalized bacteria) was quenched by exposure to trypan blue in sodium acetate buffer for 5 min. The percentage of cells phagocytosing \(M.\) tuberculosis was measured by FACS analysis. Values in A are the mean \(\pm\) SD of two independent experiments; B represents a single experiment.

Internalization of endocytic tracer

Pulse-chase experiments with FITC-labeled BSA (Sigma) were used to examine transit through the endocytic pathway. IFN-\(\gamma\)-treated and infected cells, on cell culture-treated coverslips, were pulsed for 30 min in serum-free medium containing FITC-BSA (1 mg/ml), washed, and chased for either 30 min, 1 h, or 4 h. Cells were then fixed and stained as described above with unlabeled anti-HLA-DR mAb and Texas Red-conjugated secondary Ab.

Confocal laser-scanning microscopy

A confocal laser-scanning microscope system (MRC-600; Bio-Rad Laboratories, Hercules, CA) was used to detect intracellular fluorochromes. Cells were scanned by dual excitation for FITC (green) and Texas Red (red) fluorescence. A 60× oil objective with numerical aperture of 1.4 was used and the images were captured such that the xyz dimensions were 0.2 μm. NIH Image version 1.60 was used for image analysis, and all images were based on maximal intensity projection.

Results

Phagocytosis of \(M.\) tuberculosis by THP-1 cells

Flow cytometry and FITC-labeled bacteria were used to measure phagocytosis of \(M.\) tuberculosis by THP-1 cells. THP-1 cells in suspension (10\(^6\) ml) were incubated in the presence of FITC-labeled bacteria (5 \(\times\) 10\(^9\) ml, final concentration) for 2 h and then washed extensively. Fluorescence of labeled bacteria bound to the outside of the cell was quenched by trypan blue in sodium acetate buffer. The number of cells loaded with bacteria was measured by FACS analysis. A signal is obtained only from bacteria that have been protected from quenching by internalization. As shown in Figure 1A, the proportion of phagocytic cells increased after PMA differentiation (43 vs 14%) and when bacteria were opsonized by fresh, nonimmune AB \(^+\) serum, the frequency of positivity reached 84%. As determined by FACS analysis, treatment of THP-1 cells with PMA also resulted in a substantial increase in expression of CD11b and CD14 (data not shown), both of which are considered to be potential receptors for \(M.\) tuberculosis (36, 37). PMA-differentiated cells were exposed to a range of bacterial concentrations and maximal loading with opsonized bacteria was obtained using a bacteria to cell ratio of 50:1. In contrast, undifferentiated cells exposed to the same range of nonopsonized bacteria showed only marginal internalization of bacteria (Fig. 1B). This ratio was used in all subsequent experiments. Alternatively, for each experiment, routine acid-fast staining was used to confirm the presence of bacteria.
of intracellular bacteria and the experiments were continued when the proportion of infected cells was >80%.

### Attenuation of IFN-γ-induced HLA-DR expression in THP-1 cells infected with M. tuberculosis

Initial experiments examined surface expression of HLA-DR molecules by PMA-differentiated THP-1 cells in response to incubation with a range of concentrations of IFN-γ for 36 h. FACS analysis indicated that expression of HLA-DR was maximal (MFI index 15 to 25) at 200 U/ml of IFN-γ (data not shown). This concentration was used in all subsequent experiments.

When differentiated THP-1 cells were exposed to M. tuberculosis for 24 h, cell surface expression of HLA-DR in response to IFN-γ was nearly completely abrogated (Fig. 2). In contrast, constitutive and IFN-γ-induced expression of class I molecules were unaltered by M. tuberculosis infection. The expression of two additional unrelated surface markers, CD18 and the transferrin receptor, were also unchanged in infected cells, indicating that the inhibitory effect of M. tuberculosis on MHC class II expression is selective. As shown in Table I, reduced fluorescence intensity for class II expression was first apparent at a bacteria to cell ratio of 12:1, and the extent of this reduction increased with greater multiplicities of infection.

To determine whether the attenuation of class II molecule expression is dependent on metabolically active bacteria, THP-1 cells were incubated with heat-killed (2 h, 60°C) M. tuberculosis. As shown in Figure 3, in contrast to live bacteria, phagocytosis of killed bacteria led to only a partial attenuation of HLA-DR expression (42% reduction). To control for potential nonspecific effects of phagocytosis on HLA-DR expression, cells were incubated with 0.005% latex particles (uptake of 10 to 15 particles/cell) before treatment with IFN-γ. Latex particles had no significant effect on class II induction. In contrast, incubation of cells with dextran sulfate, which is known to inhibit class II gene transcription (38), completely abrogated cell surface class II expression.

As was the case for THP-1 cells, M. tuberculosis also inhibited cell surface expression of class II molecules on monocytes obtained from normal human donors (Table II). Human monocytes appeared to be more sensitive than THP-1 cells to the effects of M. tuberculosis.

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**Table I. HLA-DR expression on THP-1 cells infected with increasing number of M. tuberculosis cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mtb</td>
<td>IFN-γ</td>
<td>Positive cells (%)</td>
</tr>
<tr>
<td>22:1</td>
<td>−</td>
<td>22.3</td>
</tr>
<tr>
<td>21:1</td>
<td>+</td>
<td>96.1</td>
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<tr>
<td>6:1</td>
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<tr>
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<td>78.4</td>
</tr>
<tr>
<td>50:1</td>
<td>+</td>
<td>28.8</td>
</tr>
</tbody>
</table>

* Differentiated THP-1 cells were incubated with opsonized bacteria (bacteria:cell ratio indicated) or with medium alone for 24 h and then IFN-γ (200 U/ml) was added for an additional 36 h. Cells were incubated for 30 min with anti-HLA-DR mAb or irrelevant isotype-matched mAb and labeling was detected by subsequent incubation with FITC-conjugated secondary Ab and FACS analysis.
FIGURE 3. Effect of killed-M. tuberculosis, latex particles, and dextran sulfate on cell surface class II expression. Differentiated THP-1 cells were incubated with opsonized live (LMB) or heat-killed M. tuberculosis (KMB), bacterialcell ratio of 50:1, with latex particles (Ltx, 0.005%) or with dextran sulfate (Dx, 150 μg/ml) for 24 h and then IFN-γ (200 U/ml) was added for 36 h. Cells were stained as described in the legend to Figure 2. Results are expressed as histograms of fluorescence intensity; solid lines represent cells stained with anti-HLA DR mAb and dashed lines represent cells stained with irrelevant isotype-matched IgG. Values in parentheses indicate MFI indices defined as in Figure 2. The data shown are representative of results obtained in three separate experiments.

tuberculosis as bacteria to cell ratios as low as 5:1 markedly inhibited cell surface expression of class II. In addition, constitutive expression of class II molecules by cultured monocytes was also diminished by infection (Table II).

M. tuberculosis effects on IFN-γ-dependent activation of the Jak-STAT pathway

The transcription of class II genes in response to IFN-γ is known to be dependent upon signaling through the IFN-γ-activated Janus tyrosine kinase, Jak1, which phosphorylates the DNA-binding protein STAT1α (19, 20, 39). To examine the possibility that attenuation of IFN-γ-induced expression of class II molecules by M. tuberculosis was related to impaired signaling through the Jak-Stat pathway, infected (for 24 h) and non-infected THP-1 cells were incubated with IFN-γ for 15 min and cell lysates were analyzed for tyrosine phosphorylation of Jak1, Jak2 and STAT1α. As shown in Figure 4, IFN-γ-induced tyrosine phosphorylation of Jak1, Jak2 and STAT1α was not influenced by infection with M. tuberculosis. These results indicate that attenuation of HLA-DR expression in M. tuberculosis-infected cells is unlikely to be explained by impaired IFN-γ-activated activation of the Jak-STAT signaling pathway.

Induction of CIITA and DRA genes is not inhibited by M. tuberculosis infection

CIITA is an essential, IFN-γ-inducible, trans-acting factor required for expression of MHC class II genes (16–18). Induction of CIITA is also known to be dependent on signaling through the Jak-Stat pathway (39). However, IFN-γ-induced expression of CIITA and DRA genes are coordinately attenuated in TGF-β-treated cells despite normal function of the Jak-Stat pathway (30). These suggested the possibility that infection with M. tuberculosis could affect CIITA expression via a mechanism independent of Jak-STAT activation. To address this question, THP-1 cells were exposed to M. tuberculosis for 24 h prior to incubation with IFN-γ (24 h). Total RNA was isolated and RT-PCR was performed using primers for CIITA and DRA. The results shown in Figure 5A demonstrate that mRNA levels for both CIITA and DRA were not influenced by infection with M. tuberculosis. In contrast, exposure of cells to dextran sulfate, which like M. tuberculosis inhibits cell surface expression of class II molecules (38), brought about marked reductions in mRNA levels for both CIITA and DRA.

To ensure that any potential inhibitory effect of M. tuberculosis on DRA induction was not obscured due to a plateau effect in the tyrosine kinase, Jak1, which phosphorylates the DNA-binding protein STAT1α (19, 20, 39). To examine the possibility that attenuation of IFN-γ-induced expression of class II molecules by M. tuberculosis was related to impaired signaling through the Jak-Stat pathway, infected (for 24 h) and non-infected THP-1 cells were incubated with IFN-γ for 15 min and cell lysates were analyzed for tyrosine phosphorylation of Jak1, Jak2 and STAT1α. As shown in Figure 4, IFN-γ-induced tyrosine phosphorylation of Jak1, Jak2 and STAT1α was not influenced by infection with M. tuberculosis. These results indicate that attenuation of HLA-DR expression in M. tuberculosis-infected cells is unlikely to be explained by impaired IFN-γ-activated activation of the Jak-STAT signaling pathway.

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To ensure that any potential inhibitory effect of M. tuberculosis on DRA induction was not obscured due to a plateau effect in the

Table II. Surface expression of HLA-DR on human monocytes infected with M. tuberculosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Positive Cells (%)</th>
<th>MFI Index</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mtb</td>
<td>IFN-γ</td>
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<tr>
<td>Donor 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mtb</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1:1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5:1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10:1</td>
<td>+</td>
<td>+</td>
</tr>
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<td>20:1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>50:1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Donor 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mtb</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>–</td>
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<td>10:1</td>
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<td>10:1</td>
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<td>+</td>
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<td>Donor 3</td>
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<tr>
<td>Mtb</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>20:1</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>20:1</td>
<td>+</td>
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To ensure that any potential inhibitory effect of M. tuberculosis on DRA induction was not obscured due to a plateau effect in the
Precipitation experiments were done using [35S]methionine-labeled cells and mAb to class II molecules. As shown in Figure 6A, three radiolabeled proteins of approximately 35 kDa (class II α-chain), 32 kDa (invariant chain, li) and 30 kDa (class II β-chain) were immunoprecipitated from lysates of IFN-γ-treated cells. In contrast, extracts of untreated cells gave only weak signals for these three labeled proteins. Importantly, the abundance of these proteins was equivalent in IFN-γ-treated control cells and in IFN-γ-treated infected cells. These findings indicate that *M. tuberculosis* does not affect the synthesis or steady state levels class II molecules.

The intracellular processing and transport of class II molecules can be estimated from the conversion of immature αβ chains to mature Endo H-resistant molecules that have undergone N-linked glycosylation during successive exposure to medial Golgi-specific enzymes. IFN-γ-treated (control and *M. tuberculosis*-infected) cells were radiolabeled and chased for various times before Endo H treatment. After a 1 h chase, the majority of class II molecules were Endo H sensitive and control and infected cells displayed similar levels of immature (indicated by α′, β′, and li′) subunits (Fig. 6B). After 8 h of chase, most class II/βi molecules were Endo H resistant (indicated by α′, β′, and li′) in IFN-γ-treated control cells and by 20 h there were no apparent Endo H-sensitive molecules. Substantial Endo H resistance also developed in *M. tuberculosis*-infected cells. However a minor proportion of Endo H-sensitive molecules persisted at 20 h of chase. Thus, infection appears to have a minimal effect on the assembly and processing of class II molecules through the Golgi and this is insufficient to explain the marked decrease in cell surface expression.

**Infection with *M. tuberculosis* inhibits maturation of class II heterodimers during post-Golgi transport to the cell surface**

Following the exit from the TGN, li chain is partially degraded in an acidic endosomal/lysosomal compartment. The residual CLIP fragment in the groove of the αβ dimer is exchanged with antigenic peptide in the MHC (22). In contrast to their immediate precursors, mature, peptide-loaded class II dimers are resistant to dissociation in the presence of SDS at room temperature, thereby enabling their specific detection (32, 33). To evaluate the maturity of class II molecules in infected cells, immunoprecipitates from IFN-γ-treated control and infected cells were incubated in 1% SDS at room temperature and analyzed by SDS-PAGE (Fig. 7). In control cells, SDS-stable molecules of the molecular mass expected for the αβ dimers (50–55 kDa) were detected at 2 h of chase and steadily increased in quantity with longer chase times, concomitant with a decrease in SDS-labile molecules. In contrast, SDS-stable complexes were barely detectable in both cells infected with *M. tuberculosis* and in cells alkalinized by treatment with NH₄Cl. These findings suggest that, if the class II/βi complex exits the Golgi and reaches the endosomal compartment in infected cells, either the enzymatic processing of li chain or the loading of αβ dimer with antigenic peptide, or both, are blocked.

**Intracellular accumulation of class II molecules in *M. tuberculosis*-infected cells**

Initially, single-color intracellular staining and epifluorescence microscopy were used to examine the presence of class II molecules in large populations of cells. As shown in Table III, for both IFN-γ-treated control and infected cells, almost 90% of cells stained positively for intracellular HLA-DR. However, while intracellular staining of control cells was relatively homogeneous throughout the cytosolic compartment, peripheral staining was negative in the majority (83 to 89%, n = 3) of infected cells indicating intracellular sequestration of class II molecules. To examine whether class II molecules are normally targeted to the endosomal compartment in infected cells, two-color immunofluorescence staining with specific Abs to HLA-DR and to Lamp-1, a marker for endosomes and...
lysosomes was performed. Cells were examined using a laser, confocal microscope. In IFN-γ-treated control cells, the staining pattern of HLA-DR (Fig. 8a, green) showed vesicles of various sizes randomly distributed throughout the cell with a notably bright peripheral staining. Class II staining overlapped significantly (yellow) with that of Lamp-1 (red). As Lamp-1 remains associated with class II vesicles en route to the surface, submembranous localization of this marker was also noted. The distribution of class II molecules in infected cells was markedly different. In contrast to control cells, infected cells showed diffuse, perinuclear staining of the cytosolic compartment and peripheral staining was negligible (Fig. 8b, green). Colocalization of class II with Lamp-1 was markedly less than that observed in IFN-γ-treated control cells. This finding suggests defective endosomal targeting of class II molecules in infected cells.

Transport of class II molecules within the endosomal compartment was examined by pulse-chase labeling of endosomes with FITC-BSA prior to intracellular staining of class II. In IFN-γ-treated control cells, internalized BSA (green) was randomly distributed throughout the cytoplasm (Fig. 8c, green). Colocalization (yellow) with HLA-DR (red) was readily observed after a 30-min chase and increased further at 1 h. After a 4-h chase, internalized BSA completely colocalized with class II molecules presumably in late endosomes and lysosomes including the MIIC. This conclusion is based on the findings that long chase periods (1 h and up) usually allow fluid-phase tracers to localize in such mature endosomal structures (40, 41). In M. tuberculosis-infected cells, internalization of FITC-labeled BSA (Fig. 8d, green) was also observed, but was much less extensive than in control cells. Notably, in contrast to control cells, colocalization of HLA-DR with BSA was negligible at both 1 and 4 h of chase. This finding indicates that the intersection of vesicles containing MHC class II molecules with late endosomal structures, including Ag-bearing endosomes, lysosomes, and the MIIC, is markedly impaired in cells infected with M. tuberculosis.

The failure of class II molecules to colocalize with endocytosed BSA may reflect an underlying defect in endocytosis and subsequent trafficking in the endosomal compartment. To examine endocytic activity, control and infected cells were incubated with

FIGURE 6. Effect of M. tuberculosis infection on the synthesis and transport of class II molecules. A. Differentiated THP-1 cells were incubated for 24 h with opsonized M. tuberculosis (Mtb) and then with IFN-γ for a further 36 h. Cells were washed and incubated with [35S]methionine for 2 h. After lysis of cells, equivalent amounts of TCA-precipitable radioactivity were subjected to immunoprecipitation with anti-HLA-DR mAb Tu36. Immunoprecipitates were boiled for 5 min in 2× sample buffer and analyzed by SDS-PAGE. The positions of m.w. markers (kDa) are indicated on the left. The electrophoretic mobilities of the α, β, and ιΙ subunits are indicated on the right. B. IFN-γ-treated (control and infected) cells were labeled (800 μCi/ml) for 30 min and chased in the presence of excess cold methionine for the indicated times. After lysis and immunoprecipitation of class II molecules, half of each sample was treated with Endo H for 24 h before SDS-PAGE. αs, βs, and ιΙs indicate Endo H-sensitive and αr, βr, and ιΙr indicate Endo H-resistant subunits. The data shown are from one of three experiments that yielded similar results.

FIGURE 7. SDS stability of αβ dimers in M. tuberculosis-infected cells. Differentiated THP-1 cells were incubated for 24 h in medium alone (Med) or with opsonized M. tuberculosis (Mtb) and then with IFN-γ for 36 h. Samples were also incubated in medium alone without any treatment (Med*). Cells were labeled (600 μCi/ml) for 30 min and chased in the presence of excess cold methionine for the indicated times. NH4Cl (10 mM) was added to selected cell samples 1 h before and during the pulse-chase with [35S]methionine. After lysis and immunoprecipitation of class II molecules, samples were held for 1 h at room temperature in 2× sample buffer before SDS-PAGE. Electrophoretic mobilities of the α, β, ιΙ subunits and the SDS-resistant αβ dimers are indicated on the right. Positions of m.w. markers (kDa) are indicated on the left.
FITC-BSA for different time periods and were analyzed by FACS. MFI indices (MFI of labeled cells/MFI of unlabeled cells) of cells internalizing FITC-BSA were calculated. Compared with control cells, infected cells showed decreased fluorescence at all time points tested (MFI index at 5 min/15 min/30 min/60 min: control = 17.0/37.3/53.1/59.4 vs infected = 2.9/4.3/18.2/33.3). These results suggest a defect in endocytosis in infected cells and may be related to abnormal trafficking of class II vesicles into the endocytic compartment. Reduced endocytic activity is consistent with an apparently less extensively developed Lamp-1 positive endosomal compartment in infected cells (compare Fig. 8b with Fig. 8a, red).

**Class II molecules intersect with vacuoles containing M. tuberculosis**

To examine directly whether class II molecules translocate to *M. tuberculosis*-containing vacuoles, cells were incubated with live or heat-killed (2 h, 60°C) FITC-labeled bacteria before addition of IFN-γ. Labeling of mycobacteria with FITC affected neither cell viability nor replication (data not shown). Moreover, as determined by FACS analysis, the effect of FITC-labeled bacteria on surface expression of class II (Table IV) was similar to that of unlabeled, viable organisms. When intracellular staining for HLA-DR and confocal laser analysis were performed, the staining pattern of HLA-DR (red) in cells incubated with killed bacteria appeared similar to that of control cells (Fig. 9a). Of note, multiple bacterial fragments (green), resulting from intracellular degradation of killed bacteria were apparent and these colocalized (yellow) with HLA-DR. In contrast, in cells infected with viable FITC-labeled *M. tuberculosis* (Fig. 9c), bright, predominantly perinuclear staining for HLA-DR (red) was observed with a periphery largely devoid of class II molecules. This pattern is qualitatively similar to that observed with cells infected with unlabeled, viable organisms (Fig. 8b). In addition, in cells incubated with FITC-labeled, live bacteria, the organisms (green) appear intact and are surrounded with substantial yellow color. These findings indicate that class II molecules colocalize with vacuoles containing either dead or live *M. tuberculosis*. However, based upon analysis in Figure 8, the distribution of class II molecules in the endosomal compartment is markedly restricted in cells harboring viable mycobacteria.

**Discussion**

The experiments reported investigated IFN-γ-induced MHC class II expression in monocytic cells infected with *M. tuberculosis*. The human cell line THP-1 (42) was used for this study because it displays many characteristics of mature monocytes and macrophages such as Ag processing and presentation to T cells (43, 44), efficient phagocytosis of *M. tuberculosis* (45) (Fig. 1), and substantial expression of class II molecules in response to IFN-γ (Fig. 2).

The results obtained show that IFN-γ-induced cell surface expression of HLA-DR molecules is markedly attenuated in THP-1 cells infected with viable *M. tuberculosis*. Inhibition of class II expression is significantly less when heat-killed bacteria are used (Fig. 3) indicating that the effects of *M. tuberculosis* on class II expression are, at least partially, dependent on metabolically active organisms. Cell surface HLA-DR expression was also diminished in human monocytes infected in vitro (Table II), consistent with data previously reported (7). Compared with THP-1 cells, exposure of monocytes to a relatively low number of bacteria resulted in high rates of infection and markedly attenuated class II expression. This relative increased sensitivity of human monocytes may be related to more abundant receptors (CD11b and CD14) for *M. tuberculosis* on monocyte plasma membranes. In contrast to diminished class II expression, both constitutive and IFN-γ-induced expression of class I molecules in the same cells were apparently increased (Fig. 2). This finding is consistent with a recent report of impaired Ag presentation through the MHC class II pathway and augmentation of MHC class I-restricted Ag presentation in *M. tuberculosis*-infected monocytes (8).

The possibility that impaired responses to IFN-γ for induction of class II expression may be related to defective cell signaling or gene expression was investigated. IFN-γ regulates the expression of class II genes primarily at the level of transcription and this requires induction of CIITA (18). Moreover, expression of the CIITA gene itself is dependent on Jak-STAT signaling (17, 39, 46). The finding that IFN-γ-induced Jak-STAT activation was not affected by infection with *M. tuberculosis* (Fig. 4) suggested the likelihood that CIITA induction should be observed. In fact, RTPCR and Northern analyses (Fig. 5, A–B) provided direct evidence that IFN-γ-induced expression of the CIITA gene occurs normally in THP-1 cells infected with *M. tuberculosis*, and mRNA levels for DRA were also observed to be unaffected. These results indicated that the level of translation or upon events involved with processing or transport of class II proteins. The findings of normal steady state levels of α, β, and Ii chains (Fig. 6A)
were consistent with effects on either the maturation or transport of class II molecules, or both.

MHC class II molecules are synthesized as nonameric complexes consisting of three $\alpha\beta$ dimers associated with a trimer of $\beta_2$-molecules ([(αβ)$_3$/β$_2$]$_3$) (22, 23, 47). After exiting the TGN, a dileucine-like motif in the cytoplasmic domain of $\beta_2$-molecules acts as targeting signal and directs (αβ)$_3$/β$_2$ complexes to an endosomal compartment. Here, $\beta_2$-molecules undergoes sequential proteolysis, giving rise to an intermediate fragment, CLIP, which remains associated with class II molecules, occupying the peptide-binding groove. This process requires an acidic pH and when completed, HLA-DM, a nonclassical MHC class II molecule, catalyzes CLIP dissociation, allowing class II molecules to bind...
antigenic peptide, prior to export to the cell surface. The cytoplasmic tail of DMB contains a tyrosine-based motif (Tyr-Thr-Pro-Leu), which directs HLA-DM molecules from the TGN to endosomes. Studies in a variety of APC types (22) suggest that removal of CLIP and peptide loading occur in a postendosomal compartment related to lysosomes, referred to as the MIIC.

Maturation and transport of class II molecules is associated with characteristic changes in their biochemical properties. Progression of class II molecules through the Golgi apparatus generates Endo H-resistant dimers that are re-istant to SDS at room temperature (32, 33). Figure 6B shows that in M. tuberculosis-infected cells, almost all radiolabeled class II molecules detected after a 20-h pulse-chase are Endo H resistant. This suggests that αβ dimers undergo normal glycosylation in the medial Golgi. However, pulse-chase analysis also revealed that M. tuberculosis inhibits the generation of SDS-resistant αβ dimers (Fig. 7). These findings suggested that M. tuberculosis may interfere with either the delivery of αβ/Ii, to the endocytic compartment or, alternatively, with the enzymatic processing of β chain and peptide loading in the MIIC. Confocal laser scanning of 0.2-μm sections demonstrated that class II molecules colocalized poorly with the endosomal/lysosomal marker Lamp-1 in infected cells. This provided direct evidence that the endosomal localization of class II/Ii chain complexes is defective in infected cells. Fluid-phase tracers of endocytosis, such as BSA, OVA, or dextran, are commonly used in pulse-chase experiments to label endosomal structures, such as lysosomes and the MIIC (40, 41, 48). Cells infected with M. tuberculosis did endocytose FITC-BSA, albeit at a significantly reduced level compared with control cells. Nevertheless, colocalization of class II molecules with internalized BSA was negligible in infected cells. This was particularly true after longer periods of chase (compare Fig. 8c to d, 1 h and 4 h of chase). These findings suggest that class II molecules exiting the Golgi do not intersect with endosomes containing internalized Ags, which is an obligatory step before progression to peptide-loaded, stable molecules in the MIIC. The absence of SDS-stable, class II dimers in infected cells (Fig. 7) is consistent with this possibility. Taken together with results from the Endo H experiments, these findings suggest that, despite normal Golgi processing, transit of newly synthesized class II molecules into the endo-cytic pathway is impaired. Diminished entry or defective transport of class II molecules in the endocytic pathway may be related to the finding of an overall reduction in endocytic activity per se.

In contrast to class II molecules, synthesis of heavy and light chains of class I molecules and peptide loading are endoplasmic reticulum-associated events. Moreover, class I/peptide complexes travel to the cell surface along a pathway that does not initially intersect the endocytic compartment (49). Since this pathway is not affected by M. tuberculosis, class I expression in infected cells does not appear to be perturbed (Fig. 2).

Based upon current information about the events involved in the regulated expression of class II molecules and results of the present study, Figure 10 provides a model that may explain the effects of M. tuberculosis on class II expression. Possible mechanisms accounting for retarded maturation of class II molecules are depicted. Metabolically active organisms secrete a variety of products including ammonia (50–52), lipoarabinomannan (LAM) (53, 54), and sulfadine (55, 56) that are believed to be virulence factors (57). Alkalization of critical intracellular organelles is a potential mechanism to explain retarded maturation of class II dimers in infected cells. Ammonia produced by M. tuberculosis, which is known to inhibit the saltatory movement of lysosomes (50, 58), may act similarly on endosomes and class II vesicles thereby inhibiting their fusion. Alternatively, ammonia produced by M. tuber-culosis may diffuse into endosomes containing class I/II complexes and prevent adequate acidification leading to inhibition of enzymes required for the processing of both Ii chain and internal-ized Ags. Consequently, immature class II molecules may be retained in the endocytic pathway. These hypotheses are supported by the finding that treatment of cells with NH4Cl, like M. tuber-culosis, also inhibits the generation of SDS-resistant αβ dimers.

**Table IV. Surface expression of HLA-DR in THP-1 cells incubated with FITC-labeled M. tuberculosis**

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>MFI</th>
<th>Anti-DR Ab</th>
<th>Irrelevant Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.19b</td>
<td>4.39</td>
<td></td>
</tr>
<tr>
<td>Unlabeled, live</td>
<td>0.20</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>FITC-labeled, live</td>
<td>0.25</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td>FITC-labeled, killed</td>
<td>0.20</td>
<td>3.10</td>
<td></td>
</tr>
</tbody>
</table>

* Differentiated THP-1 cells were incubated with opsonized bacteria (bacteria:cell ratio, 50:1) or with medium alone for 24 h and then IFN-γ (200 U/ml) was added for an additional 24 h. Cells were incubated for 30 min with anti-HLA-DR mAb or irrelevant isotype-matched mAb, and labeling was detected by subsequent incubation with phycoerythrin-conjugated secondary Ab and FACS analysis.

* MFI of 5000 events scattered on live cells.

![FIGURE 9](http://www.jimmunol.org/) Colocalization of M. tuberculosis with class II molecules. Adherent cells were incubated in medium alone (a) or incubated with opsonized killed (b) or live (c) FITC-labeled M. tuberculosis (bacteria:cell ratio of 50:1) and then treated with IFN-γ for 24 h. Cells were stained with anti-DR mAb and Texas Red-labeled secondary Ab and analyzed as described in Figure 8. The green color shows bacteria and the red color shows HLA-DR. The yellow signal depicts colocalization. Bar, 10 μm.
In summary, the data presented indicate that: 1) *M. tuberculosis* markedly attenuates the cell surface expression of class II molecules in response to IFN-γ, 2) activation of the Jak-STAT signaling pathway and expression of CIITA and DRA in response to IFN-γ are not inhibited in *M. tuberculosis*-infected cells, 3) class II molecules are synthesized in infected cells and transit the medial Golgi in an apparently normal manner, but fail to colocalize with late endosomal/lysosomal organelles, and 4) retardation of intracellular transport and maturation of HLA class II molecules by *M. tuberculosis* prevents their transit to the cell membrane. These findings provide a basis for investigating products of *M. tuberculosis* that interfere with the maturation/processing of MHC class II molecules leading ultimately to inhibition of class II-restricted Ag presentation.

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References


